Why do frog and toad forelimbs suddenly (but asynchronously) appear every time metamorphosis is near?

Why do frog and toad forelimbs suddenly (but asynchronously) appear every time metamorphosis is near?

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Short title: Why do forelimbs suddenly appear, every time metamorphosis is near?

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Summary

1. The internal development and emergence of the forelimbs at metamorphosis is a defining feature of anuran amphibians (frogs and toads). However, although forelimb emergence is considered sudden, it is rarely synchronous. Any asynchrony may or may not exacerbate the increased drag that is predicted to occur with the emergence of the forelimbs at metamorphic climax.

2. Despite the impact forelimb emergence is hypothesized to have on individual survival and life history evolution, the degree of asynchrony between forelimb emergence, and any consequences of such asymmetry, have not been investigated. The asynchrony in forelimb emergence also provides an opportunity to test the currently held evolutionary basis for the internal development and sudden emergence of the forelimbs in anurans.

3. Using a diverse range of anuran taxonomic groups we measured the time between, and pattern of, emergence of the forelimbs across a range of species. To examine the evolutionary impacts of forelimb emergence we assessed locomotory performance when individuals had zero, one or two forelimbs emerged.

4. The duration of time between the emergence of the two forelimbs was longer and more variable than predicted. Furthermore, no species suffered impaired burst speeds nor was their angle of escape affected as the forelimbs emerged asynchronously. In fact, burst swimming speed was faster after the emergence of one and two forelimbs than prior to their emergence.

5. Fundamentally, our results call into question the proposition, long accepted, that internal forelimb development is associated with locomotion and reducing drag during metamorphosis. This does not appear to be the case and we suggest that anatomical or developmental constraints or advantages may be responsible.

Key words: anuran amphibian, asymmetry, constraints, development, forelimb emergence, life history, locomotory performance, metamorphic climax

Introduction

Tetrapod forelimb development is highly diverse (Polly 2007), yet some larval anuran amphibians (the tadpoles of frogs and toads) are unique in having delayed development of the forelimbs relative to the hind limbs (Bininda-Emonds *et al.* 2007), internal development of the forelimb, and sudden eruption of the well-developed limb through the outer body layer. In fact, the sudden emergence of the developed forelimbs is a distinguishing developmental characteristic of the group and represents a significant life history event, concluding larval development, and is often used to demarcate the onset of the rapid transition to the adult form (metamorphic climax) (Gosner 1960; Walsh 2010). During metamorphosis, the eruption of the forelimbs, in particular, has been viewed as critical since it noticeably and immediately changes the shape of the tadpole.

In anurans, the forelimbs commonly develop within the branchial chamber – a space containing the gills – enclosed laterally by the operculum (analogous to the gill covers of bony fishes) and that links to the exterior via an opening called the spiracle. Less commonly, as in the pipids (clawed frogs, e.g. *Xenopus laevis*), the forelimbs develop within brachial sacs, structures that are separate from and posterior to the opercular cavity (Newth 1948). The position of the spiracle or spiracles differs among taxonomic groups and has been linked to forelimb emergence (e.g. Borkhvardt & Malashichev 1997). In tadpoles where the spiracle is lateral, the forelimb on the spiracular side emerges via enlargement of the spiracle. On the other side, and in tadpoles where the spiracle is central or the forelimbs are not enclosed by the opercular cavity, the forelimb emerges through a new perforation in the overlying tissue (Newth 1949). Formation or expansion of the openings for the forelimbs occurs through thinning of the overlying tissues in response to rising thyroid hormone levels, and mechanical pressure from the underlying limb (Helff 1926; Helff 1939; Newth 1949).

74 Understanding of the evolutionary processes responsible for the internal development
75 and sudden emergence of the forelimbs is poor. Wassersug (1989) argued that the forelimbs
76 develop internally and emerge suddenly because of the cost of drag (Wassersug 1989;
77 Dudley, King & Wassersug 1991) increasing predation risk (Wassersug & Sperry 1977),
78 absence of need for lateral appendages for turning (achieved by the tail), and the need for
79 morphologically distinct forelimbs at the time of metamorphosis. However, although the two
80 forelimbs emerge abruptly and fully-formed, their emergence is asymmetric (Malashichev
81 2002). Furthermore, to date the temporal separation between emergence of the two forelimbs
82 has not been quantified, and the locomotory performance impacts of forelimb emergence
83 asymmetry have not been assessed. Here we quantify this asymmetry in a taxonomically
84 diverse set of six anuran species, and test the widely assumed, but largely unexplored,
85 hypothesis that internal development and sudden emergence of anuran forelimbs in anurans
86 occurs in order to minimise reduction in locomotory performance.

87 We explored the impact of limb emergence on burst swimming speed – a key trait in
88 larval escape from predators (Van Buskirk & McCollum 2000). As tadpoles progress from
89 no forelimbs emerged, to one emerged, to both emerged, the impact of drag could have two
90 potential outcomes. The "limb drag" hypothesis proposes that drag from the limbs would be
91 cumulative, such that burst swimming speed would decrease linearly with number of
92 emerged limbs, but would not necessarily impact turning performance (Fig. 1). There would
93 also be limited selective pressure to minimize the period of asynchrony between the
94 forelimbs emerging, since in terms of locomotion, having one emerged limb is half as bad as
95 having both emerged. The "locomotion disruption" hypothesis proposes that drag from the
96 limbs would disrupt the normal swimming of the tadpole, in a manner analogous to dragging
97 an oar on only one side of a boat. Therefore it is predicted that burst speed would sharply
98 decrease after one forelimb has emerged, but with a smaller incremental decline (or

potentially a recovery) when both forelimbs are exposed (Fig. 1). This second hypothesis further predicts that turning behaviour would be biased, and show an increased acuteness in the angle of escape, in the direction of the first limb to emerge (Fig. 1). Under this scenario, we would also expect that the period between forelimbs emerging would be short and relatively invariable. Both hypotheses predict that burst swimming performance would be best without any emerged forelimbs (Wassersug & Sperry 1977).

Materials and methods

Study species

We quantified limb emergence asymmetry and burst swimming in six anuran species. The common frog *Rana temporaria* (Ranidae) and common toad *Bufo bufo* (Bufonidae) are found throughout Europe. Both have aquatic tadpoles and terrestrial adults and a single spiracle on the left side of the body (Helff 1939; McDiarmid & Altig 1999). The oriental fire-bellied toad *Bombina orientalis* (Bombinatoridae) is a primarily aquatic species from central Eastern Asia whose tadpoles have a single spiracle on the ventral midline (McDiarmid & Altig 1999). The three species of clawed frogs (Pipidae: *Xenopus laevis*, *Xenopus borealis*, *Silurana tropicalis*) are from sub-Saharan Africa and remain fully aquatic as adults. The tadpoles all have a pair of symmetrically placed lateral spiracles (McDiarmid & Altig 1999).

Animals and rearing conditions

Wild *Rana temporaria* and *Bufo bufo* eggs were collected near Glasgow, Scotland. *Xenopus laevis* (University of St. Andrews, Scotland), *Xenopus borealis*, and *Silurana tropicalis* (The Fish Hut, Lanarkshire, Scotland) were obtained as fertilised eggs from captive-bred stock. *Bombina orientalis* were obtained as late stage tadpoles from a private breeder. All tadpoles apart from *B. orientalis* were reared similarly. Tadpoles were reared in aerated, de-

chlorinated copper-free water at 21 ± 3°C and a 12:12 L:D photoperiod. In 2008, in an attempt to space the timing of metamorphosis by varying development rates, tadpoles were allocated to tanks at different densities, ranging from one to 10 individuals per litre of water. The majority of tadpoles used were reared at 10 tadpoles (57.7%) or 5 tadpoles (36.5%) per litre of water, the remaining 5.7% were reared at 1 tadpole or 2.5 tadpoles per litre. Density did not affect the body size of individuals used in the study (SVL: $F_{3,51} = 0.34$, $P = 0.80$; mass: $F_{3,51} = 0.15$, $P = 0.93$). In 2011, all individuals were reared at the same density of approximately 5 tadpoles per litre. Water was changed as required and tadpoles were fed daily *ad libitum*; *R. temporaria*, *B. bufo* and *B. orientalis* were fed on commercial fish flakes (Aquarian Tropical Flake Food, Mars Fishcare, Inc.) and the three pipids were fed on Tetra Wafer Mix (Tetra, Melle, Germany) algal pellets.

Tadpoles were observed daily until the first tadpoles approached metamorphic climax; thereafter tanks were inspected two to four times daily for individuals that had reached the stage prior to forelimb emergence, which were removed. These individuals were not provided with food, because tadpoles do not feed during metamorphic climax and this was usually reached within 48 hours. The study was conducted across two years, 2008 and 2011.

In 2008 all individuals (13 *X. laevis*, 22 *X. borealis* and 17 *S. tropicalis*) found before any limb emergence were used to examine emergence asynchrony (Observational Study; see below). In 2011, individuals that were found before one forelimb had emerged were randomly allocated to either the Observational study or the Experimental study (see below). No tadpoles were used in both studies. The Observational study used 17 *R. temporaria* and 20 *B. bufo* tadpoles; the Experimental study used 25 *R. temporaria*, 25 *B. bufo*, 16 *X. laevis* and 16 *B. orientalis*. Snout-vent length (SVL; ± 0.1 mm) for all individuals was measured using a single digital photographic frame taken with a Photron FASTCA-PCI camera and Motion Tools software (Photron USA, Inc., San Diego, California, United States).

149

150 **Observational study: Variation in forelimb asynchrony**

151 Tadpoles were placed individually in a 10 cm diameter glass dish filled *c.* 1 cm deep,
152 sufficient to cover the body and allow free swimming but restricting tadpoles to a horizontal
153 posture. Water was kept at 22°C (± 2°C). Data were recorded using filming under
154 continuous light, using a miniature charge-coupled device (CCD) CCTV camera placed
155 directly above the observation dish, such that the entire dish was in view. After both
156 forelimbs emerged, individuals were allowed to complete metamorphosis and returned to the
157 site or location from where they were obtained. Examination of video footage allowed
158 determination of the interval between first and second forelimb emergence to a resolution of
159 ±1 min.

160

161 **Experimental study: Burst speed and direction of escape**

162 Burst swimming speed and escape direction were quantified at three developmental points: 1)
163 just prior to the emergence of either forelimb; 2) with one forelimb emerged; and 3) within 12
164 hours after the second forelimb had emerged and before significant reduction in tail length.
165 At each stage, tadpole burst swimming speed and direction were measured five times with *c.*
166 1 min interval between each. The side that the first forelimb emerged from and SVL,
167 following the methods above, were recorded.

168 Tadpoles were placed individually in the centre of a 30 cm swimming arena, as
169 described above and allowed to settle for 2 minutes. Burst swimming was initiated by a
170 consistent discharge of air from a 1 ml Gilson pipette to the rear of the animal (Van Buskirk
171 & McCollum 2000). All trials were recorded in the same room, under the same conditions,
172 that tadpoles experienced during rearing using a Photron FASTCAM-PCI high-speed camera

placed 50 cm directly over the arena. Laminated grid-paper was placed under the arena to allow calibration of distances in video analysis.

Filming was carried out at 250 frames per second (fps) for up to 5 s of swimming in each trial. As elsewhere (Watkins 1996; Dayton *et al.* 2005; Wilson, Kraft & Van Damme 2005; Walsh, Downie & Monaghan 2008a; Walsh, Downie & Monaghan 2008b), the first 300 ms following initial movement were used to estimate burst speed (Fig. 2), calculated as the distance travelled (cm) per second. The angle of escape was calculated by determining the grid coordinates of the snout and the vent in the frame prior to initial movement and the grid coordinates of the same two points in the last frame. From this the orientation of the individual before and after moving and the change in angle from the starting position was calculated, using the general formula: $\text{Angle} = 2 * \arctan\left(\frac{y}{\sqrt{x^2+y^2}+x}\right)$, where y is the distance the snout moved along the y-axis and x is the distance the snout moved along the x-axis. This was then subtracted or added, depending on the orientation of the tadpole, from the starting angle of orientation of the tadpole determined from the same formula, but where y is the distance on the y-axis between the snout and vent and x is the distance on the x-axis between the snout and vent. The direction of the turn was also recorded as either left or right.

Data analysis

All analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, Il, U.S.A.). A general linear model (GLM) was used to examine burst speed, with SVL as a covariate, species as a factor, tadpole ID as a random factor and the number of forelimbs emerged (either zero, one or two) and trial as repeated measures. Escape direction was analysed with generalized estimating equations (GEE), using a binary logistic model. Species was included as a factor, trial and number of emerged forelimbs as repeated measures, and the first forelimb to emerge (either left or right) as a covariate. To analyse the angle of escape a linear mixed model

198 (LMM) was used, with species as a fixed factor, trial and the number of emerged forelimbs as
199 repeated measures, tadpole ID as a random factor, and the first forelimb to emerge as a
200 covariate. All significant interactions were included in all models.
201

202 ## Results

203 ### Forelimb emergence

204 The time taken between the emergence of the first and second forelimb was highly variable in
205 all five species investigated (Table 1). Within the 1 minute resolution of the study, there was
206 no case of simultaneous forelimb eruption. The period between emergence of the forelimbs
207 did not differ amongst the five species ($F_{4,80} = 0.39$, $p = 0.82$), the density the individuals
208 were reared at ($F_{3,80} = 0.30$, $p = 0.47$), nor did it depend on which forelimb emerged first
209 ($F_{1,80} = 1.24$, $p = 0.27$).
210

211 ### Locomotory performance

212 In contrast to the predictions of our two hypotheses, all four species tested for burst speed
213 swam *slowest* prior to the emergence of either forelimb ($F_{2,1179.86} = 8.60$, $P < 0.001$; Fig. 3).
214 The four species differed in their overall burst speed ($F_{3,103.09} = 13.46$, $P < 0.001$). Of the
215 four species, *R. temporaria* exhibited the fastest increase in burst speed with the emergence
216 of the first forelimb ($F_{6,1170.02} = 32.12$, $P < 0.001$) such that they swam fastest with only one
217 forelimb emerged (post-hoc $P < 0.001$; Fig. 3) compared to when no and both forelimbs were
218 exposed. SVL, which decreased as the forelimbs emerged (Fig. 4), did not consistently affect
219 burst speed ($F_{1,318.86} = 0.47$, $P = 0.49$). However, there was a significant interaction between
220 SVL and the number of forelimbs emerged ($F_{2,1180.91} = 6.78$, $P < 0.005$), with burst speed
221 getting faster as SVL increased when one or both forelimbs emerged, but not when neither

forelimb had emerged. Trial did not significantly affect burst speed ($F_{4,1132.92} = 2.32$, P = 0.06).

Individuals from all four species tested had a right-bias in turning after being startled ($\chi^2_1 = 56.21$, $p < 0.0001$; Fig. 5). Species differed in the magnitude of this bias (Wald $\chi^2_3 = 11.81$, $p = 0.008$) consistently over the five trials (Wald $\chi^2_4 = 1.59$, $p = 0.81$). Within species, the strength of rightwards bias decreased as tadpoles progressed from no forelimbs to both forelimbs emerged (Wald $\chi^2_2 = 12.09$, $p = 0.002$). However, contrary to the locomotion disruption hypothesis, the first forelimb to emerge did not affect the direction that individuals turned on escaping (Wald $\chi^2_1 = 0.56$, $p = 0.46$).

There was no overall change in the angle of escape with the progression through forelimb emergence ($F_{2, 785.66} = 2.66$, $p = 0.07$). Species differed in their responses ($F_{3, 77.23} = 4.69$, $p = 0.005$) and responded differently when either no, one, or both forelimbs had emerged ($F_{6, 752.78} = 4.92$, $p < 0.0001$; Fig. 6). Individuals appeared to escape at a steeper angle, regardless of direction, when the left forelimb, rather than the right, was the first to emerge but this was marginally non-significant ($F_{1, 75.86} = 3.85$, $p = 0.05$). The trial did not affect the angle of escape ($F_{4,416.81} = 1.26$, $p = 0.29$).

Discussion

Our results do not support either the limb drag or locomotion disruption hypothesis, or the general idea that external forelimbs influence swimming performance and hence predation risk (Wassersug & Sperry 1977; Wassersug 1989). We observed no decrease in burst speed during the progression of forelimb emergence (counter to both hypotheses), no direct predicted bias or impairment of turning during escape (counter to the locomotion disruption hypothesis), and a long and highly variable period between the emergence of the forelimbs (counter to the locomotion disruption hypothesis). Our results thus re-awaken the debate

247 over why anuran forelimbs develop internally and erupt suddenly at the start of metamorphic
248 climax.

249 There are several possible explanations for our findings. Firstly, it is possible that
250 limb emergence does influence burst speed and ease of turning, but that we did not observe
251 any effects due to compensation for increased or asymmetrical drag forces by the
252 metamorphs (Schmidt-Nielsen 1972; Webb 1988; Hildebrand & Goslow Jr. 2001; Azizi &
253 Landberg 2002; Landberg & Azizi 2010). Evidence for such investment would imply that
254 there is a direct cost to impaired swimming performance. Utilising more energy to maintain
255 burst performance during forelimb emergence could deplete energy stored for
256 metamorphosis, affecting survival in subsequent encounters and size on completion of
257 metamorphosis with associated knock-on effects (Altwegg & Reyer 2003; Chelgren *et al.*
258 2006).

259 Similarly, we may not have observed a decrease in burst speed because burst speed is
260 based on physiological capacity and motivation (Losos, Creer & Schulte 2002), and our
261 methodology might not have provoked a consistent level of motivation across different
262 stages. While motivation may differ across the different stages examined in this study, the
263 maximum burst speeds for *Xenopus laevis* (18.9 - 27.7 cm s^{-1}) from our current study are
264 comparable with previous studies on this species (Wilson, James & Johnston 2000: *c.* 30 cm
265 s^{-1}; Walsh *et al.* 2008a: 19.6 - 24.6 cm s^{-1}). Directly comparable results are not available for
266 the other species, yet *Bombina orientalis* from our study were substantially faster (21.8-27.0
267 cm s^{-1}) than early stage (Gosner stage 20-21) *B. orientalis* tadpoles (Kaplan & Phillips 2006:
268 4.9 - 5.5 cm s^{-1}), as would be expected. Therefore, our methodology does appear to elicit an
269 appropriate response.

270 Alternatively, the drag force imposed by exposed forelimbs, calculated by Dudley *et*
271 *al.* (1991) to be less than that presented by the externally developing hind limbs, may not

represent a significant impediment to locomotion. The forelimbs, after erupting, can be held close to the body during swimming, rather than in a protracted position (Dudley *et al.* 1991), lessening their drag. Furthermore, forelimb emergence should not only be regarded as a potential cost, since it is central to the opportunity, unavailable to limbless tadpoles, of moving on land and flexibility in the timing of the transition onto land appears beneficial (Downie, Bryce & Smith 2004; Touchon *et al.* 2013).

To be effective, the degree and direction of turning when escaping a predator should be somewhat unpredictable (Domenici, Blagburn & Bacon 2011), but could be biased by the presence of a single exposed forelimb. However, having only one forelimb emerged did not bias the direction that individuals turned (i.e. individuals with only the left forelimb emerged did not predominantly turn left, as expected under significant drag). Similarly, forelimb asynchrony did not appear to impair their ability to turn, nor did it exaggerate or dampen the angle of escape. Interestingly, we also did not observe a change in the angle of escape when both forelimbs were present as might be expected from having lateral appendages available for steering (Wassersug 1989).

Though our hypotheses make only qualitative predictions about the extent of limb emergence asynchrony (that it should be minimal), we saw relatively substantial differences in emergence times of the two limbs. It has been argued that the fully aquatic life history of pipid clawed frogs may limit any costs of drag caused by emergence of forelimbs (Walsh *et al.* 2008a). The pipid species did indeed demonstrate the greatest degree of variability (CoV > 90%). However, species with terrestrial adults – the frog *R. temporaria* and the toad *B. bufo*, whose tadpoles more closely resemble the generalised form considered by Wassersug (1989) and Dudley *et al.* (1991), also showed high variability (CoV ≥ 75%). One suggestion is that any cost of forelimb emergence asymmetry might be avoided by behavioural modification during metamorphic climax (Touchon *et al.* 2013), limiting selection for

297 synchronised emergence. Anurans do not feed during metamorphosis and may alter their
298 behaviour or choices of microhabitat to remain sheltered from predators (Ydenberg & Dill
299 1986; Lima & Dill 1990; Skelly 1994; Downie *et al.* 2004), rather than relying on escape
300 responses. Examining the behaviour of metamorphosing individuals for these species - in
301 particular, activity levels, microhabitat selection, and when during metamorphic climax
302 individuals make the transition between habitats - would be required to explore any impact of
303 forelimb synchronisation (Touchon *et al.* 2013).

304 However, more significantly, not only did all species not show a decrease in burst
305 speed, they actually swam faster after the emergence of one forelimb than before, regardless
306 of whether their adults are predominantly aquatic (*X. laevis* and *B. orientalis*) or terrestrial (*R.*
307 *temporaria* and *B. bufo*). Similarly, with the exception of one study (Wassersug & Sperry
308 1977), locomotory impairment during metamorphic climax has not been observed at all
309 (Watkins 1997; Walsh *et al.* 2008a; Walsh *et al.* 2008b) or occurs only after forelimb
310 emergence, associated with re-absorption of the tail (e.g. post-Gosner (Gosner 1960) tadpole
311 development stage 43: (Huey 1980; Brown & Taylor 1995)). Overall, these results seriously
312 question the proposition that locomotory impairment from the exposed forelimb explains
313 their internal development and sudden eruption (Wassersug 1989). Therefore, another
314 explanation is needed.

315 One possibility is that the internal development and sudden eruption may represent a
316 simple developmental constraint (Smith *et al.* 1985) of the lineage. For example, in the
317 common coqui *Eleutherodactylus coqui*, which develops directly into its adult form within an
318 egg, the forelimbs are enclosed by the opercular fold and emerge from this tissue via
319 perforations similar to species that undergo a metamorphosis (Callery & Elinson 2000).
320 However, there are no data on this phenomenon in other direct developing frogs.
321 Alternatively, it may represent an anatomical constraint, for example, of having the forelimbs

322 developing outside the body in the vicinity of the branchial chamber. Comparative studies
323 with urodele amphibians (newts and salamanders), whose tadpoles commonly have external
324 gills and develop forelimbs externally, may prove useful. Thirdly, selection for the delayed,
325 internal development and sudden eruption of the forelimbs could occur during different life
326 stages not examined in this study. Similarly, the selection for internal development of the
327 forelimbs may not relate to heightened predation risk caused by drag from the exposed limb,
328 but from exposed limbs being vulnerable to, or presenting additional targets for, predators
329 (Ballengee & Sessions 2009). Finally, there may be a developmental advantage in retaining
330 the forelimbs within the body cavity as they develop. While the forelimbs seemingly have no
331 function in the developing tadpole, they are essential for the juvenile and adult frog
332 (Wassersug 1989). Therefore their protection during the larval stage and rapid development
333 approaching metamorphosis may be advantageous. We hypothesise that retaining the
334 forelimbs within the body cavity may make it easier to both elevate and regulate their
335 temperature for rapid development (Casterlin & Reynolds 1978). This would coincide with
336 anuran amphibians' peak thermal preference, which occurs as their forelimbs develop and
337 just before they emerge (Dupré & Petranka 1985), and is compatible with the finding by
338 Bininda-Emonds *et al.* (2007) that amongst the tetrapod vertebrates studied, anuran
339 amphibians are unique in having forelimb development retarded relative to hind limb
340 development.
341

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348

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Table legends

Table 1: Mean, median, range and coefficient of variance of time taken between the emergence of first and second forelimb. All times are presented in minutes.

Figure legends

Fig. 1 Graphical representations of the predictions for the "limb disruption" (circle) and "limb drag" (square) hypotheses. Filled shapes relate to the burst speed axis and open shapes to the escape direction bias axis. The dotted lines indicate the alternative possibility that burst performance may improve with the emergence of the second limb. For the escape direction bias axis, 0.0 represent no directional bias in turning, negative values indicate turning in the opposite direction from the first limb to emerge, and positive values indicate turning in the same direction as the first exposed limb

Fig. 2 Composite image from video footage of tadpole burst swimming. Images of the tadpole are taken from just prior to initiating burst response and every 60 ms after the first sign of movement. Burst speed was measured as the distance the snout travelled from the initial position to the final position over the 300 ms

Fig. 3 Mean (±SE) absolute (a) and relative (b) burst speed of the four different species, with no forelimbs, one forelimb and both forelimbs emerged (open circle: *Rana temporaria* (N = 25); closed circle: *Bufo bufo* (N = 25); closed triangle: *Xenopus laevis* (N = 16); open triangle: *Bombina orientalis* (N = 16)). Relative burst speed is presented to allow comparisons among the different sized species used in this study

Fig. 4 The mean (±SE) snout-vent length of the four different species, with no forelimbs, one forelimb and both forelimbs emerged that were used for assessing burst speed (open circle: *Rana temporaria*; closed circle: *Bufo bufo*; closed triangle: *Xenopus laevis*; open triangle: *Bombina orientalis*. Sample sizes as in Fig. 3

Fig. 5 The turning bias after being startled in the four different species, with no forelimbs, one forelimb and both forelimbs emerged. Positive values indicate a right bias and negative values a left bias, 0.0 represents no bias (open circle: *Rana temporaria*: closed circle: *Bufo bufo*; closed triangle: *Xenopus laevis*; open triangle: *Bombina orientalis*). Sample sizes as in Fig. 3

Fig. 6 The angle of escape away from swimming directly forward in a straight line, which would be represented by 0°, of the four different species, with no forelimbs, one forelimb and both forelimbs emerged (open circle: *Rana temporaria*: closed circle: *Bufo bufo*; closed triangle: *Xenopus laevis*; open triangle: *Bombina orientalis*). Sample sizes as in Fig. 3

502 Table 1

Species	N	Mean time (± SE)	Median time	Time range	CoV
R. temporaria	17	354.1 ± 64.4	349.0	11 – 847	74.9 %
B. bufo	20	362.8 ± 68.3	246.0	10 – 913	84.2 %
X. laevis	13	544.6 ± 142.0	264.0	29 – 1433	94.0 %
X. borealis	22	280.8 ± 63.7	193.0	2 – 1348	106.4 %
S. tropicalis	17	320.2 ± 82.5	231.0	1 – 1477	106.2 %

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